RADIATION-STIMULATED DNA SYNTHESIS IN CULTURED MAMMALIAN CELLS

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ABSTRACT

A type of DNA synthesis in mammalian cells that is stimulated by ultraviolet light has been studied by means of radioautography and density gradient centrifugation. The characteristics of this synthesis are: (*a*) it is not semiconservative; (*b*) it is enhanced by the presence of 5-bromodeoxyuridine in the DNA molecule; (*c*) the degree of stimulation is dose dependent; (*d*) there is less variability in the rate of incorporation of H^3-thymidine during this synthesis than during normal DNA synthesis; (*e*) it occurs in cells that are not in the normal DNA synthesis phase (G_1 and G_2 cells). This kind of synthesis has been found in cultured cell lines from five different species; however, in some strains, the presence of bromouracil in the DNA is required before it can be demonstrated by radioautography.

INTRODUCTION

We have reported that ultraviolet light (UV) causes a type of DNA synthesis in cultured mammalian cells which may be indicative of a repair process (1). When HeLa S3 cells are exposed to UV light and then incubated with tritiated thymidine (H^3-TdR), all of the cells show uptake of the tracer into DNA within 30 min or less. This phenomenon also occurs in Chinese hamster cells, strain DFAF-33, but only when they have been previously grown in medium containing 5-bromodeoxyuridine (5-BUdR). Quantitative measurements of the uptake of radioactive nucleic acid precursors show that at moderately high doses of UV light (above an incident dose of about 200 ergs/mm^2) both HeLa S3 and DFAF-33 cells incorporate increasing amounts of thymidine with increasing dose, provided they have been previously grown in medium containing 5-BUdR. On the basis of these findings, we have hypothesized that this may be evidence for a system that actively repairs UV damage to DNA in mammalian cells. The present communication contains data on DNA synthesis following UV irradiation which are consistent with this hypothesis.

It seems reasonable that a repair system, if it is to be efficient, should recognize various types of damage. Also, if it conferred a competitive advantage, it might be widely distributed through many species. Accordingly, we have reexamined the behavior of HeLa cells after X-irradiation, and also made a survey of a number of cell lines derived from different species in regard to their incorporation of H^3-TdR into DNA after exposure to UV light.

MATERIALS AND METHODS

Cell Lines

HeLa S3 (obtained from Dr. R. Drew, Brookhaven National Laboratory, Upton, New York) and DFAF-33 cell lines (obtained from Dr. G. Yerganian

Childrens Cancer Research Foundation, Boston, Massachusetts) have been routinely maintained in Eagle's medium in our laboratory.

In our laboratory DFAF-B was isolated from a culture of DFAF-33 that was grown continuously in the presence of 5-BUdR 5 μg/ml. Incorporation of exogenous thymidine, cytidine, or 5-BUdR into its DNA is not detectable under our conditions.

Cell line CH46101, derived from the Chinese hamster, was obtained from the laboratory of Dr. E. H. Y. Chu, (Oak Ridge National Laboratory, Oak Ridge, Tennessee).

Strains CBL and ENDO were derived from rabbit central nervous system tissue, and were obtained from the laboratory of the late Dr. Charles M. Pomerat, (Pasadena Foundation for Medical Research, Pasadena, California).

All other strains were obtained as frozen cultures from the American Type Culture Collection, Washington, D. C.

CsCl Density Gradient Studies

Cell cultures were grown in plastic Petri dishes (Falcon Plastic Company, Los Angeles). After treatment appropriate to the experiment, the cells were harvested by scraping the cells from the dishes into SSC (0.15 M NaCl, 0.015 M sodium citrate) and by centrifuging the suspension. The resulting pellet was resuspended in about 2 ml of SSC, and several drops of 1% sodium dodecyl sulfate were added by continuous stirring. The lysate was shaken vigorously for about 1 min with an equal volume of chloroform–isoamyl-alcohol (24:1). The resulting emulsion was centrifuged at 2000 *g* for 20 min. The volume of the upper layer was adjusted to 3.0 ml with SSC, and added to 3.900 g of purified CsCl. The solution was placed in a 5-ml cellulose acetate tube, layered over with mineral oil, and centrifuged in the Spinco SW-39 rotor at 37,000 RPM for 48 hr. The tube bottom was then punctured, 2-drop samples collected separately, and each diluted with 0.5 ml of SSC. The radioactivity was determined by counting aliquots in a liquid scintillation spectrometer. The DNA was rebanded by pooling those samples containing the DNA and adding a 3.0 ml portion to an amount of CsCl calculated to bring the total CsCl in the sample to approximately 3.9 g, then by centrifuging as before. About 100 to 120 drops were collected, but all data were normalized to 100 drops for graphing.

Radioautographic Experiments

Irradiation conditions, labeling with H^3-TdR, and film application were the same as previously described (1). In most of the experiments reported here, the cells were stained according to the Feulgen reaction before application of the stripping film. After development, crystal violet (0.025% was used as a counterstain. In some cases, duplicate cultures were treated with DNase for 1 hr at room temperature at pH 6.3.

Ultraviolet Light Source

A Mineralight lamp (Ultraviolet Products, Inc., San Gabriel, California) delivering the bulk of its energy at 2537 A was arranged to give a dose rate of about 5 ergs/mm² per sec.

X-Ray Experiments

The cells were grown on cover slips in Leighton tubes. For irradiation, the tubes were placed on a rotating turntable in an unfiltered X-ray beam. The source was a General Electric 300 KV Maxitron. The dose rate as measured by a Victoreen ionization chamber was about 300 rad/min, under conditions of minimal scatter. After irradiation, the cells were incubated in Eagle's medium containing H^3-TdR at 10 μc/ml for 60 min, then fixed and stained as in the case of the UV experiments.

RESULTS

Radioautographic Experiments

Incorporation of H^3-TdR by all HeLa cells after exposure to UV light is detectable at very low doses. Cover slip cultures were grown for 48 hr with either 5-BUdR (5 μg/ml) or TdR (4 μg/ml); duplicates were then exposed to UV doses in the range of 15 to 120 ergs/mm² before incubation with H^3-TdR. Radioautograms showed that at these relatively low doses a considerable amount of DNA synthesis still occurred in those cells which were in S phase, in addition to the UV-induced uptake of H^3-TdR in cells in other stages of the growth cycle. This is illustrated in Fig. 1. Fig. 1 *a* is a photomicrograph of a radioautogram of unirradiated HeLa cells grown for 48 hr with 5-BUdR at 5 μg/ml and then incubated for 30 min with H^3-TdR at 10 μc/ml. Fig. 1 *b* is of a similar culture irradiated with 30 ergs/mm² prior to incubation with H^3-TdR. In both cases, two groups of cells are apparent. The heavily labeled cells are those which were in S phase at the time of incubation with H^3-TdR, and the fraction of these is the same in both normal and irradiated populations. In the unirradiated population, the other cells are free of label, but the irradiated cells show a uniform light labeling over the nuclei of those cells which are not in S phase, but (presumably) in G_1 or G_2.

It is of interest that this UV-induced uptake of

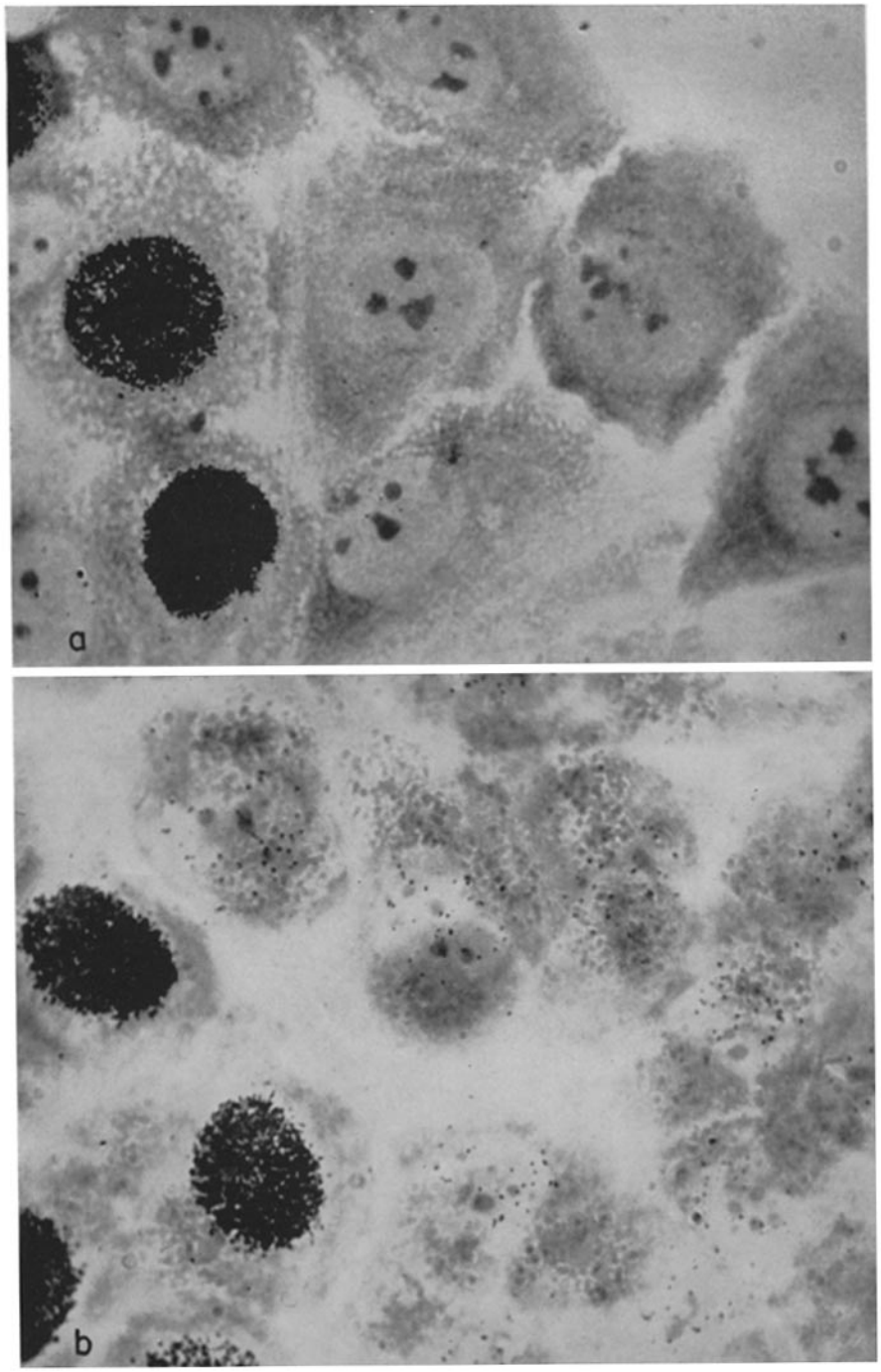

FIGURE 1 Photomicrographs of radioautograms of HeLa S3 cells which were grown for 48 hr in Eagle's medium containing 5-BUdR 5 μg/ml, UV-irradiated (or not) with 30 ergs/mm^2, and incubated in fresh medium containing H^3-TdR at 10 μc/ml (6.7 c/mM) for 30 min; (1*a*) control, (1*b*) irradiated. × 972.

H^3-TdR also occurs in cells in mitosis. An example is shown in Fig. 2. These are HeLa cells which were given a relatively high dose of UV light (1500 ergs) and incubated with H^3-TdR as described above. At this dose, cells in S phase are indistinguishable from cells in other phases, because the UV has essentially abolished all normal DNA replication. In the center of the field is a dividing cell in late anaphase or telophase showing heavy label over the chromosomal regions. Labeling has been observed in all stages of mitosis. This observation is most interesting since it indicates that the DNA molecule is accessible for participation in this UV-stimulated uptake of H^3-TdR, even though the chromosomes are in a highly condensed state.

Table I shows the dose dependence of the uptake of H^3-TdR in the form of counts of the number of silver grains appearing over the nuclei of cells in G_1 or G_2 after various UV doses. There are a number of points of interest in these data. First, the number of grains per nucleus increases with increasing doses of UV light. Second, those cells grown in medium containing 5-BUdR show higher mean grain counts for the same dose of UV than those grown with TdR. Fig. 3 is a plot of the data from Table I, in which the mean grain counts are plotted against the log of the dose. The reason for straight lines resulting from this type of plot is not clear, but the marked difference between the slopes of the curves clearly shows that the presence of 5-BUdR sensitizes the DNA molecule to UV light, as far as this phenomenon is concerned. A third point is the low variance of the grain counts of the lightly labeled cells. The ratio $S^2/\bar{X}$ for these counts does not exceed about 3, whereas grain counts with comparable means obtained from cells which incorporated H^3-TdR during normal DNA synthesis exhibit $S^2/\bar{X}$ ratios of 10 or more (2).

CsCl Density Gradient Studies

The density gradient studies of Pettijohn and Hanawalt (3) on the repair of UV-damaged cells in bacteria indicate that in this system the process is of a "cut and patch" nature; i.e., the damaged

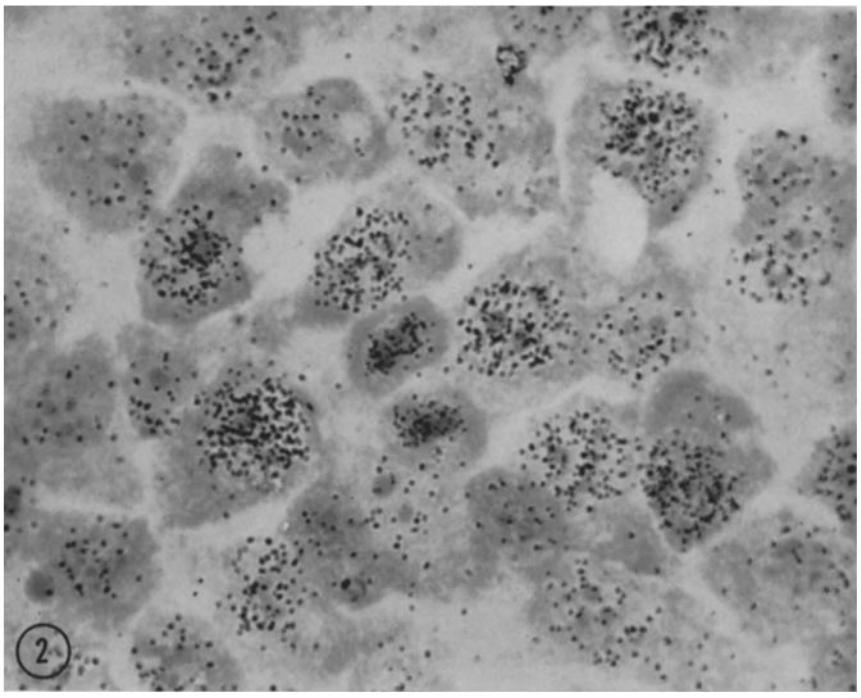

FIGURE 2 Photomicrograph of a radioautogram of HeLa S3 cells given a UV dose of 1500 ergs/mm² and incubated with H^3-TdR at 10 μc/ml for 30 min. Note the labeled mitotic figure in the center of the field. × 972.

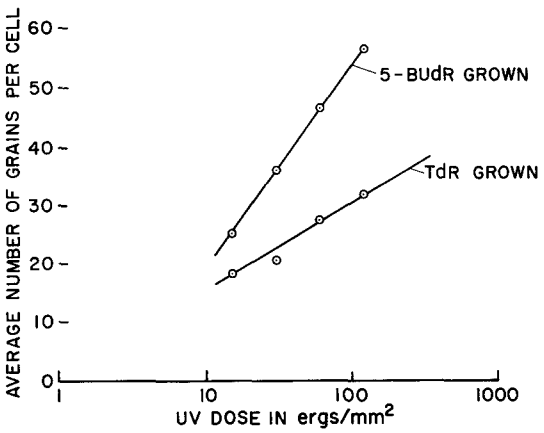

FIGURE 3 Dose dependence of grain count in HeLa S3. Plot of the grain count data from Table I.

area of the DNA is removed and replaced. Using a similar approach, we have found evidence that a process similar to the "patch" action exists in mammalian cells, but we have not been able to demonstrate prior excision of regions of the DNA. Fig. 4 shows the results of an experiment in which HeLa cells were grown for approximately one generation in Eagle's medium with 2-C^{14}-5-BUdR at 5 μg/ml and 0.3 μc/ml, irradiated with 1000 ergs/mm^2, and incubated in Eagle's medium containing H^3-TdR at 1 μc/ml (14 c/mM) for 3 hr. The high UV dose was used essentially to abolish normal DNA replication. Duplicate cultures were similarly treated except for the irradiation. Fig. 4 *a* is the result obtained when the DNA from unirradiated HeLa cells was banded in a CsCl gradient. The 2-C^{14}-5-BUdR serving as a density label clearly distinguished the hybrid DNA formed prior to the incubation with H^3-TdR. The pattern of tritium activity shows that it was incorporated into material banding at both the hybrid position and at a lighter position, corresponding to normal density DNA. Since semiconservative replication of hybrid density DNA in the absence of a density label yields DNA of both hybrid and normal density, the pattern conforms to expectation. The higher incorporation into the light DNA is probably due to replication by DNA molecules that had not yet incorporated BUdR during the prior incubation. Fig. 4 *b* is the pattern obtained with DNA from the cells which were irradiated prior to incubation with H^3-TdR. The formation by tritium of a separate, less dense peak is not observed, but there is still considerable tritium activity incorporated into the DNA which bands at the hybrid position; thus, this incorporation does not represent semiconservative replication. Figs. 4 *c* and *d* are the patterns obtained when the fractions containing the DNA indicated in 4 *a* and *b*, respectively, were pooled and banded a second time in a CsCl gradient with normal nonradioactive HeLa DNA included as a marker. The hybrid DNA from the irradiated cells bands at a position corresponding to a lower density than that from unirradiated cells. This has been observed in other similar experiments as well.

TABLE I

Effect of UV on the Uptake of H^3-TdR into HeLa Cells Not in S Phase

Preirradiation medium supplement	UV dose	Data from grain counts of 25 cells at each dose		
		$\bar{X} \pm S_{\bar{X}}$	S^2	$S^2/\bar{X}$
	ergs/mm²			
5-BUdR (5 μg/ml)	15	26.0±1.3	45	1.7
	30	36.8±1.5	64	1.7
	60	46.7±2.4	140	2.9
	120	56.3±2.4	139	2.4
	Control	≃5		
TdR (4 μg/ml)	15	18.6±1.1	32	1.5
	30	20.3±1.6	62	3.1
	60	27.6±1.9	89	3.2
	120	32.0±2.0	98	3.1
	Control	≃3		

The possibility that the tritium activity in the hybrid peak was not in DNA, but rather some low molecular weight compound that was associated with the DNA, has been effectively ruled out by the following experiment. HeLa cultures were grown, irradiated, and incubated with H^3-TdR as above. Half of the DNA extracted from the

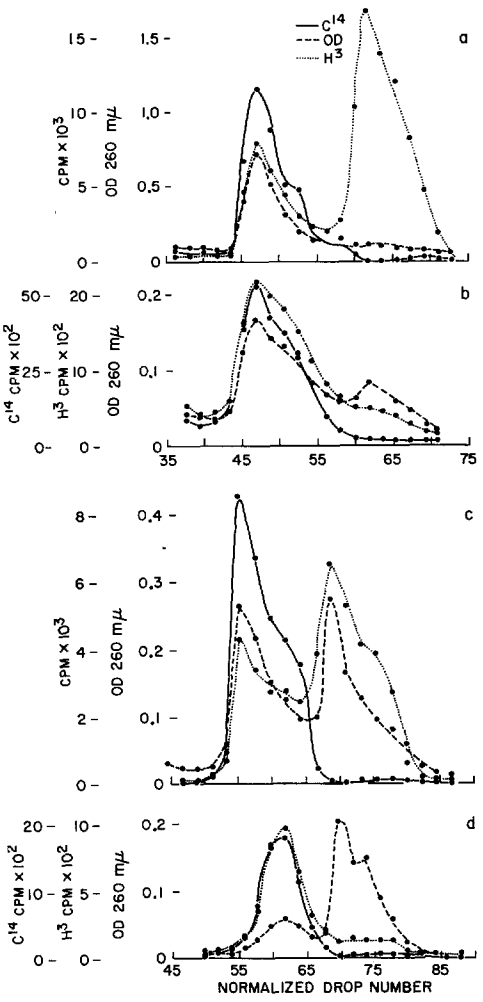

FIGURE 4 CsCl density gradient analysis of DNA from HeLa S3 cells. The cells were grown for 18 hr in medium containing 2-C^{14}-5-BUdR 5 μg/ml (0.3 μc/ml). Following irradiation with 1000 ergs/mm² of 2537 A UV light, they were incubated with fresh medium containing H^3-TdR (1 μc/ml, 14 c/mM) for 3 hr; (*4a*) unirradiated control, (*4b*) irradiated, (*4c* and *d*) control and irradiated rebanded with added unlabeled HeLa DNA as a marker.

irradiated cells was loaded on a 1 × 47 cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) and eluted with SSC. The DNA emerged very quickly since it was not retarded by the Sephadex, and it was then analyzed in a CsCl gradient, as was the DNA not put through the column. The results showed the same ratio of tritium activity to C^{14} activity in the hybrid peak of treated material as in the DNA not treated with Sephadex. If this tritium activity were not in material of high molecular weight, it would have been retarded on the column and not come through with the DNA, unless it was unusually tightly bound.

A second kind of density gradient experiment consisted of incubating the cells in Eagle's medium containing H^3-TdR for 24 hr, irradiating the cells, and then incubating the cells for 3 hr in medium containing C^{14}-BUdR. The results of banding the DNA from such cells in CsCl are shown in Fig. 5. Fig. 5 *a* is the pattern obtained from unirradiated cells. Here, the DNA synthesized with C^{14}-BUdR appears in a separate, denser band than the preexisting DNA. This result is expected on the basis of semiconservative replication. Note that the C^{14} counts are at a minimum when the H^3 counts reach a maximum. Fig. 5 *b* is the pattern of DNA from cells irradiated with UV (1000 ergs/mm²) prior to incubation with C^{14}-BUdR. The bulk of DNA, as indicated by H^3 activity and absorbance, still bands at the normal position. The C^{14} activity, however, is not localized.

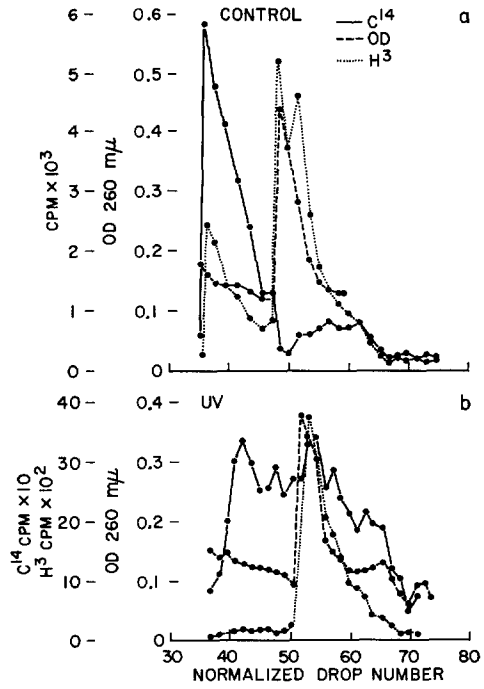

FIGURE 5 CsCl density gradient analysis of DNA from HeLa S3 cells grown for 24 hr in medium containing H^3-TdR at 1 μc/ml (6.7 c/mM), irradiated (or not) with 1000 ergs/mm² and incubated in medium containing 2-C^{14}-5-BUdR at 5 μg/ml (0.3 μc/ml) for 3 hr; (*5a*) control, (*5b*) irradiated.

TABLE II

Survey of Various Cell Lines for Incorporation of H^3-TdR after Exposure to UV Light

Cell line	Species of origin	% Labeled cells*			
		TdR grown		BUdR grown	
		Control	Irradiated	Control	Irradiated
Chang Liver	Human	38	99+	24	99+
L-132	"	44	100	9	94
Hep-2	"	27	100	27	100
J-111	"	67	89	35	90
B-14-FAF-28-G-3‡	Chinese hamster	25	33	43	96
Don	" "	27	99	43	93
CH46101	" "	42	98	44	99
DFAF-B	" "	0	0	0	0
CBL	Rabbit	30	99+	34	99+
ENDO	"	23	99+	14	99+
NCTC Clone 2472	Mouse	52	56	62	97
CCRF S-180 II	"	31	33	36	86
LLC-MK_2	Monkey	49	95	46	98

* Based on counts of at least 500 cells.

‡ This line was the parent strain of DFAF-33. The experimental data for the latter were presented previously (1).

Instead, it is spread over a range of densities, with small maxima in the normal and hybrid region. Rebanding of the material in a second CsCl gradient has confirmed these maxima, but the C^{14} appearing at the light end of the normal DNA did not reappear.

Survey of Cell Lines for UV-Stimulated Uptake of H^3-TdR

The results of the survey are shown in Table II. Every cell line tested, except one, showed almost 100% incorporation of H^3-TdR into DNA after growth in medium containing 5-BUdR and exposure to UV light, and many showed the phenomenon when grown in normal medium. The failure of strain DFAF-B to show the effect in either case is due to its inability to use exogenous thymidine. Indeed, this can be considered further evidence that in the other cell types an active incorporation into DNA occurs, rather than a passive association of the label with DNA.

X-Ray Experiments

Cover slip cultures of HeLa cells were exposed to 5000 rad of X-radiation and incubated with H^3-TdR as in the case of the UV-irradiated cells. Radioautograms showed that essentially all cells incorporated detectable tritium activity into the DNA (Fig. 6). The labeling pattern is similar to that observed after low doses of UV, showing that normal DNA synthesis is still quite active even in these heavily X-irradiated cultures. This resistance of normal DNA synthesis to X-rays has precluded density gradient studies to determine whether the synthesis (exhibited by non-S cells) is semi-conservative.

DISCUSSION

There are two major requirements for a DNA repair system: (*a*) the damage must be recognized and either removed or rendered innocuous; (*b*) the proper sequence of bases must be restored. In bacterial systems, the first of these has been demonstrated (4, 5), but evidence for the second, while very suggestive, is still circumstantial. This evidence consists in showing the presence of "repair" systems which excise damaged sites in the DNA molecule and instigate subsequent non-conservative replication in radiation-resistant organisms and the absence of such systems in radiation-sensitive organisms (3). It should be noted that the repair response can also be elicited by factors other than radiation. Hanawalt and Haynes (6) have shown that nitrogen mustard will affect the DNA of radiation-resistant bacteria in such a manner that the subsequent DNA synthesis resembles very closely that in irradiated cells, i.e., it could be interpreted as a cut and patch repair.

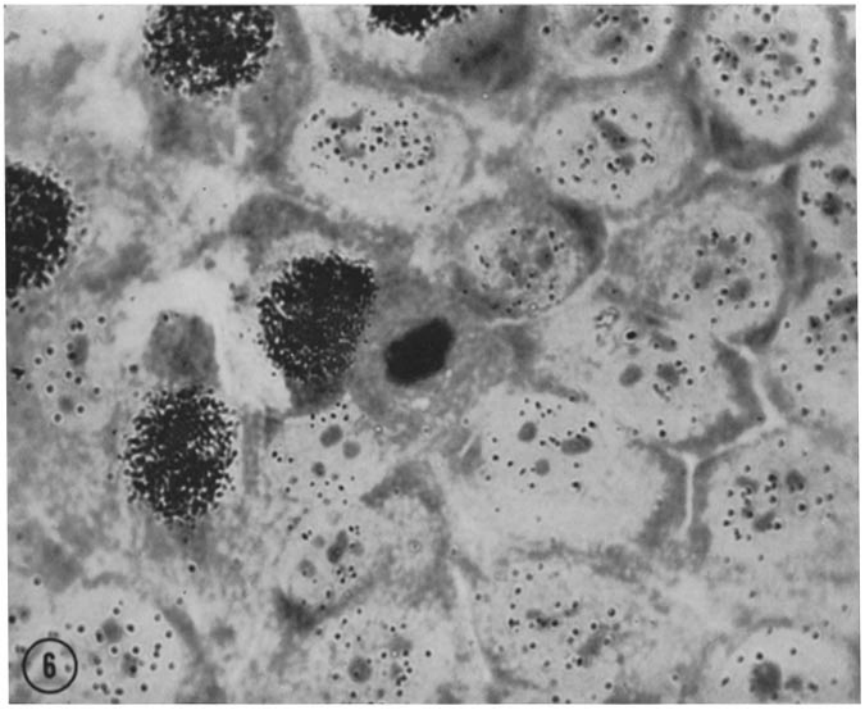

FIGURE 6 Photomicrograph of a radioautogram of HeLa cells exposed to 5000 rad of X-rays and incubated with H^3-TdR at 10 μc/ml (6.7 c/mM) for 60 min. × 972.

Mammalian cells have certain disadvantages if one attempts to do analogous experiments on them. Because of their relatively long generation time and the difficulty of dealing with large numbers of cells, the selection of mutant strains is a problem. Consequently, we have thus far restricted our investigations to those phenomena characteristic of repair that occur in established cell lines. To date, the removal of damaged regions of the DNA molecule in mammalian cells has not been observed, even though some of the UV photoproducts may be the same in both bacteria and mammalian cells (7). Trosko et al. have reported that UV-induced thymine dimers in Chinese hamster DNA do not disappear from the DNA, even after 24 hr (7). This seems to reflect a difference in the two systems as far as dealing with UV damage is concerned. However, this may be more apparent than real, since models for excision of damaged DNA without its degradation can easily be constructed. UV light, even at very low doses, stimulates the uptake of H^3-TdR into the DNA of HeLa cells not undergoing normal DNA synthesis. At all doses less than 120 ergs/mm^2, 50% or more of HeLa S3 cells survive (8), making a repair hypothesis especially attractive.

The incorporation of 5-BUdR into DNA has been shown to sensitize many organisms to radiation, including cultured mammalian cells. In this instance, the presence of 5-BUdR enhances the incorporation of H^3-TdR after UV exposure. This fact, together with the dose dependence of the H^3-TdR uptake, indicates that the extent of incorporation depends directly on the amount of UV damage, and so provides further circumstantial evidence for a repair process.

Some cell strains showed the effect only after being grown in medium with 5-BUdR. There is no readily apparent explanation for their different response. It is possible that the mechanism initiating the response is only sensitive to the greater damage occurring after irradiation of BU-DNA in these cells. It does not appear to be a species difference since, of three tested strains derived from the Chinese hamster, one required growth with 5-BUdR to show the phenomena and two did not.

Comparison of the variance of the grain counts observed in this case (Table I) with that in the

case of H^3-TdR incorporation during normal synthesis (2) shows that it is much lower in the present instance, indicating that the observed UV-stimulated uptake is similar in all cells and more closely resembles the kinetics of a single rate reaction than does normal DNA synthesis.

Density gradient studies show that when cells are labeled with C^{14}-BUdR before irradiation (Fig. 4), the irradiated cells exhibit incorporation of H^3-TdR only into the hybrid DNA, with little evidence of labeled normal density DNA, which would result from semiconservative replication. Additionally, the density of the hybrid DNA of the irradiated cells is apparently reduced (Fig. 4 *d*). It is not possible to tell from these data whether the reduction in density is partially due to removal of portions of the DNA molecule containing 5-BUdR, or entirely to the addition of thymidine, since either action would change the proportion of the two bases. Both of these points, nonsemiconservative synthesis and reduced density of the DNA from irradiated cells, are consistent with a patch mechanism. Other possible explanations for the observed uptake of H^3-TdR after UV irradiation, such as initiation of new "growing points" (9, 10) in the chromosome or side chain production of the type observed with the Kornberg system (11), do not seem likely, since the uptake appears to have some specificity for thymidine (1).

In the experiment in which the cells were incubated with H^3-TdR before irradiation and with C^{14}-BUdR afterward (Fig. 5), the expected result, on the basis of 5-BUdR substituting for thymidine in the repair process, would be that the C^{14} activity would be concentrated in the light peak. This is partially borne out by the presence of a C^{14} peak underlying the H^3 light peak, but the nonhomogeneous density distribution of C^{14} activity is difficult to account for. It is possible that some of the C^{14} in denser material is the result of residual normal DNA synthesis.

Finally, although the data quite clearly show that UV light initiates a nonsemiconservative kind of DNA synthesis in mammalian cells, there is no evidence that it is really a repair phenomenon. Under the restrictions of presently developed techniques, it seems almost impossible to demonstrate that the observed response leads to greater viability. Therefore, the next best approach, as in bacteria, would be to attempt to correlate the presence or absence of this response with radio resistance or radio sensitivity, respectively. Such experiments are being planned.

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